Possible role of Cdx2 in the serrated pathway of colorectal cancer characterized by BRAF mutation, high-level CpG Island methylator phenotype and mismatch repair-deficiency

Dawson, Heather ; Galván, José A ; Helbling, Melina ; Muller, Dominique-Elisabeth ; Karamitopoulou, Eva ; Koelzer, Viktor H ; Economou, Mary ; Hammer, Caroline ; Lugli, Alessandro ; Zlobec, Inti

Abstract: Colorectal cancer is a heterogeneous disease at the histomorphological, clinical and molecular level. Approximately 20% of cases may progress through the "serrated" pathway characterized by BRAF mutation and high-level CpG Island Methylator Phenotype (CIMP). A large subgroup are additionally microsatellite instable (MSI) and demonstrate significant loss of tumor suppressor Cdx2. The aim of this study is to determine the specificity of Cdx2 protein expression and CpG promoter hypermethylation for BRAFV600E and high-level CIMP in colorectal cancer. Cdx2, Mlh1, Msh2, Msh6, and Pms2 were analyzed by immunohistochemistry using a multi-punch tissue microarray (TMA; n = 220 patients). KRAS and BRAFV600E mutation analysis, CDX2 methylation and CIMP were investigated. Loss of Cdx2 was correlated with larger tumor size (P = 0.0154), right-sided location (P = 0.0014), higher tumor grade (P < 0.0001), more advanced pT (P = 0.0234) and lymphatic invasion (P = 0.0351). Specificity was 100% for mismatch repair (MMR)-deficiency (P < 0.0001), 92.2% (P < 0.0001) for BRAFV600E and 91.8% for CIMP-high. Combined analysis of BRAFV600E/CIMP identified Cdx2 loss as sensitive (80%) and specific (91.5%) for mutation/high status. These results were validated on eight well-established colorectal cancer cell lines. CDX2 methylation correlated with BRAFV600E (P = 0.0184) and with Cdx2 protein loss (P = 0.0028). These results seem to indicate that Cdx2 may play a role in the serrated pathway to colorectal cancer as underlined by strong relationships with BRAFV600E, CIMP-high and MMR-deficiency. Whether this protein can only be used as a "surrogate" marker, or is functionally involved in the progression of these tumors remains to be elucidated.

DOI: https://doi.org/10.1002/ijc.28564

IJC
International Journal of Cancer

Possible role of Cdx2 in the serrated pathway of colorectal cancer characterized by BRAF mutation, high-level CpG Island methylator phenotype and mismatch repair-deficiency

Heather Dawson[1,2†], José A. Galván[2], Melina Helbling[2], Dominique-Elisabeth Muller[2], Eva Karamitopoulou[1,2], Viktor H. Koelzer[1,2], Mary Economou[2], Caroline Hammer[2], Alessandro Lugli[1,2] and Inti Zlobec[2]

[1] Department of Clinical Pathology, Institute of Pathology, University of Bern, Bern, Switzerland
[2] Translational Research Unit, Institute of Pathology, University of Bern, Bern, Switzerland

Colorectal cancer is a heterogeneous disease at the histomorphological, clinical and molecular level. Approximately 20% of cases may progress through the "serrated" pathway characterized by BRAF mutation and high-level CpG Island Methylator Phenotype (CIMP). A large subgroup are additionally microsatellite instable (MSI) and demonstrate significant loss of tumor suppressor Cdx2. The aim of this study is to determine the specificity of Cdx2 protein expression and CpG promoter hypermethylation for $BRAF^{V600E}$ and high-level CIMP in colorectal cancer. Cdx2, Mlh1, Msh2, Msh6, and Pms2 were analyzed by immunohistochemistry using a multi-punch tissue microarray (TMA; $n = 220$ patients). KRAS and $BRAF^{V600E}$ mutation analysis, CDX2 methylation and CIMP were investigated. Loss of Cdx2 was correlated with larger tumor size ($P = 0.0154$), right-sided location ($P = 0.0014$), higher tumor grade ($P < 0.0001$), more advanced pT ($P = 0.0234$) and lymphatic invasion ($P = 0.0351$). Specificity was 100% for mismatch repair (MMR)-deficiency ($P < 0.0001$), 92.2% ($P < 0.0001$) for $BRAF^{V600E}$ and 91.8% for CIMP-high. Combined analysis of $BRAF^{V600E}$/CIMP identified Cdx2 loss as sensitive (80%) and specific (91.5%) for mutation/high status. These results were validated on eight well-established colorectal cancer cell lines. CDX2 methylation correlated with $BRAF^{V600E}$ ($P = 0.0184$) and with Cdx2 protein loss ($P = 0.0028$). These results seem to indicate that Cdx2 may play a role in the serrated pathway to colorectal cancer as underlined by strong relationships with $BRAF^{V600E}$, CIMP-high and MMR-deficiency. Whether this protein can only be used as a "surrogate" marker, or is functionally involved in the progression of these tumors remains to be elucidated.

Cancer Genetics

In 1999, the group of Issa *et al.* defined a novel phenotype in colorectal cancer based on widespread CpG island methylation: the CpG Island Methylator Phenotype (CIMP).[1] In colorectal cancer and other tumor types, this aberrant methylation of promoter region CpG islands is associated with transcriptional inactivation of tumor suppressor genes, and is intimately involved in tumor progression.[2–4]

Despite variations in methodologies for the assessment of CIMP such as differences in gene panels and methods of analysis,[5] most studies have shown an incremental worsening in prognosis in patients with high-level CIMP.[6–10] At the clinical and histomorphological level, CIMP-high colorectal cancers tend to occur more frequently in the right colon.[11] They may have more mucinous histology and are poorly differentiated.[12,13] Association of CIMP-positivity with some environmental factors has been identified such as cigarette smoking and folic acid intake, the latter suggesting a possible interaction with 5-FU-based therapies.[14,15] Indeed, CIMP status has also been explored as a putative predictive biomarker in several studies; high-level CIMP may have a positive benefit in patients treated with 5-FU-based chemotherapy.[16–18]

Most striking about CIMP-high colorectal cancers are their associations at the molecular level with BRAF mutation,[13,19,20] CDKN2A (p16INK4A)[21,22] and microsatellite instability (MSI).[20,23] These relationships have led to the proposal of different models of colorectal cancer progression based on mutation, methylation and MSI. In one such model, ~20% of colorectal cancers are thought to arise through the serrated pathway. BRAF mutation in a first step is thought to

Key words: colorectal cancer, Cdx2, CIMP, BRAF, serrated pathway
Additional Supporting Information may be found in the online version of this article.
Author Contributions: Histopathology review of all cases was performed by HD, VHK, AL and EK. HD and EK additionally evaluated all immunohistochemistry. MH and DEM performed all molecular analysis. ME and CH provided technical expertise and performed immunohistochemistry staining. JAG performed *in situ* hybridization, scoring and statistical analysis. IZ conceived the study and study design, data interpretation, statistical analysis, and manuscript drafting. All authors reviewed, edited and approved the final version of the manuscript.
Grant sponsor: Novartis Foundation for Biomedical Research and Bernese Cancer League
DOI: 10.1002/ijc.28564
History: Received 31 July 2013; Accepted 10 Oct 2013; Online 26 Oct 2013
Correspondence to: PD Inti Zlobec, PhD, Murtenstr. 31, CH-3010 Bern, Switzerland, Tel.: 0041-31-6328755, Fax: 0041-31-6324995, E-mail: inti.zlobec@pathology.unibe.ch

What's new?
Some colorectal cancers have aberrant methylation of CpG islands, mutations in the BRAF gene, and deactivation of the tumor suppressor Cdx2. Previous studies have shown that patients with CpG methylation do worse than those without. This study sought to determine whether loss of Cdx2 also means poorer outcomes, and how it correlates with CpG methylation and BRAF mutation. Without Cdx2, they found, tumors grew larger and attained a higher grade; loss of Cdx2 also strongly predicted BRAF mutation and CpG methylation, and methylation of the CDX2 gene correlated with Cdx2 protein loss. Cdx2 might, therefore, play a role in spurring colorectal cancer.

induce normal epithelium to undergo an initial cellular proliferation which is followed by senescence.[24] In a second step, oncogene-activated cell senescence may be overcome by methylation-induced silencing of CDKN2A or other mechanisms such as p53 loss leading to the development of a preneoplastic lesion, *i.e.*, the sessile serrated adenoma, or serrated polyp and then finally to cancer.[25] Importantly, among the relevant tumor suppressor genes frequently silenced by CIMP is MLH1, a critical gene involved in DNA mismatch repair. When hypermethylated, MLH1 contributes to the development of MSI, a feature observed in 15% of all colorectal cancers. Minoo and Jass could show that BRAF mutation was causally related to MSI in colon cancer cell lines.[26] Most recently, the existence of the serrated pathway as a consequence of BRAF mutation and direct relationship to MSI was shown *in vivo* using a novel BRAF-mutant mouse model of intestinal pathology.[27]

A particular feature of MSI-high colorectal cancers is its relationship to Cdx2, a homeodomain transcription factor which functions to regulate intestinal epithelial cell differentiation.[28] Because the expression of Cdx2 is almost entirely restricted to the gastrointestinal tract, it is used as a marker for the identification of tumors of intestinal origin in daily diagnostic routine.[29] However, our group and others have demonstrated that mismatch repair (MMR)-deficient or MSI-high colorectal cancers have significant losses of Cdx2 expression.[30] Furthermore, Cdx2 loss has been associated with more aggressive histomorphological features, unfavorable survival time and could act as a marker for both $BRAF^{V600E}$ mutation and CIMP-high status.[13,31,32]

On the basis of these findings, we hypothesize that loss of Cdx2 expression is specific to tumors characterized by $BRAF^{V600E}$ mutation and CIMP-high (and therefore by extension MSI-high) and therefore may serve as a marker for the serrated pathway. The aim of this study is to determine whether Cdx2 loss is associated with adverse clinicopathological features and is specific for MSI, $BRAF^{V600E}$ mutation and CIMP-high status. In a second aim, we investigate CDX2 hypermethylation as a possible cause of reduced Cdx2 expression.

Patients and Methods

Patients

The patient cohort consisted of 220 patients with primary colorectal cancers treated at the Fourth Department of Surgery, University of Athens Medical School in Athens, Greece, between 2002 and 2007. Complete histopathological and clinical information was retrospectively collected for each patient including pT, pN, pM, tumor grade, histological subtype, venous invasion and lymphatic invasion. TNM staging was performed according to the 6th ed. of the AJCC/UICC manual. Tumor budding was assigned as low- or high-grade according to a reproducible method.[33] Survival time and information on adjuvant therapy was available. No patient received preoperative therapy. Overall median survival time for the cohort was 60 months.

Specimen characteristics

Formalin fixed (10% neutral buffered formalin) paraffin-embedded tumor blocks were retrieved from the corresponding institute of pathology. One representative tumor block was identified for tissue microarray (TMA) construction, immunohistochemistry and subsequent DNA extraction. Additionally, 17 cases of sessile serrated adenomas (SSA) or tubular adenomas (TAs) were selected and re-reviewed. These included 7 SSAs, 9 TAs with low-grade dysplasia, and 1 mixed case of TA with a small focus of high-grade dysplasia (5%) /SSA. Ethical consent was obtained from the local ethics commission.

Cell lines

In addition to colorectal cancer tissues, eight well-established human colon cancer cell lines were included in this study (HCT15, SW620, LS174, LS180, SW480, HCT116, COLO205, HT29). Cells were harvested after trypsinization in a solution of 0.05% of Trypsin-EDTA and washed two times in PBS-buffer. Four drops of serum were added to the cell sediment and mixed to dissolve the pellet. One drop of thrombin was then added to the solution and incubated for 2 min at room temperature until a clot was formed. The clot was transferred into a plastic cassette and incubated in 4% formalin. After dehydration in graded alcohols and immersion in xylene, paraffin-embedding of each cell line was undertaken and a cell block was made. A TMA containing two punches per cell lines was constructed (total = 16 cores).

Immunohistochemistry

Immunohistochemistry for Cdx2 as well as for MMR proteins Mlh1, Msh2, Msh6, and Pms2 was performed on the multi-punch tissue microarray with an average of four tumor cores per patient. Protocols for all stains have been

previously described.[34] For Cdx2, the average percentage of expression was determined across all tumor punches and ranged from 0 to 100%. MMR protein expression was defined as positive when any staining in any tumor punch was observed. Negativity for a MMR protein was defined when all tumor punches for that marker were 0%. A patient was then classified as MMR-deficient when at least one of the four proteins was negative. Because information on family history was unavailable, no attempt was made to further subdivide patients into Lynch syndrome or sporadic MSI. Cdx2 immunohistochemistry was additionally performed on the cell line TMA and on all 17 adenoma cases.

CDX2 mRNA *in situ* hybridization

mRNA *in situ* hybridization (ISH) for CDX2 was performed on the colorectal cancer patient TMA using probes from RNA Scope® (VS Probe-Hs-CDX2, NM_001265.4, probe region: 901-1803, Advanced Cell Diagnostics, Hayward, CA) on an automated platform (Discovery Ultra, Ventana, Rotkreuz, Switzerland). A negative control using DapB (dihydrodipicolinate reductase gene) mRNA expression of Bacillus subtilis and a "moderately" expressing positive control PPIB (peptidylprolyl isomerase B) were used. The following score was employed to determine the semi-quantitative expression of CDX2 mRNA per TMA punch: score 0 = no staining, score 1 = difficult to see under 40×, score 2 = difficult to see under 20× but easy under 40×, score 3 = difficult to see under 10× but easy under 20× and score 4 = easy to see under 10×.

Molecular analysis

DNA extraction. DNA was extracted from each primary colorectal cancer. First, the corresponding Hematoxylin and Eosin (H&E) stained slides were reviewed and areas of tumorous tissue were marked. A minimum of five whole tissue sections from each case were cut from corresponding paraffin-embedded tissue blocks at 4 μm. Slides were scratched using a scalpel in designated regions. DNA was extracted using standard protocols (FFPE Kit, Qiagen).

Mutation and methylation analysis by pyrosequencing. All analyses were carried out using the pyrosequencing method. BRAF (exon 15, V600E mutations) and KRAS (exon 2, codon 12 and 13) mutations were interrogated. For CpG methylation analysis, bisulfite conversion of all DNA samples was undertaken using the EpiTect Bisulfite Conversion Kit (Qiagen) according to manufacturer's instructions. The Hs_CDX2_03 assay from Qiagen containing forward and reverse (biotinylated) primers was used for the investigation of CDX2 hypermethylation. The sequence to analyze was 5′-GGCGACGGGGGCGCGCCCCGT-3′ and contained 5 CpG sites located at Chr 13:28541900-28542023. A representative pyrogram for this CDX2 assay is illustrated in Figure 1*a*. Amplification by PCR was performed using 45 cycles at 56°C. CpG analysis for SOCS1, NEUROG1, MLH1, CRABP1A, CDKN2A, and RUNX3 were also carried out using 45 amplification cycles at 56°C for all primers except NEUROG1 (57°C). Primer sequences are listed in Supporting Information Table 1. Following PCR, fragment analysis was

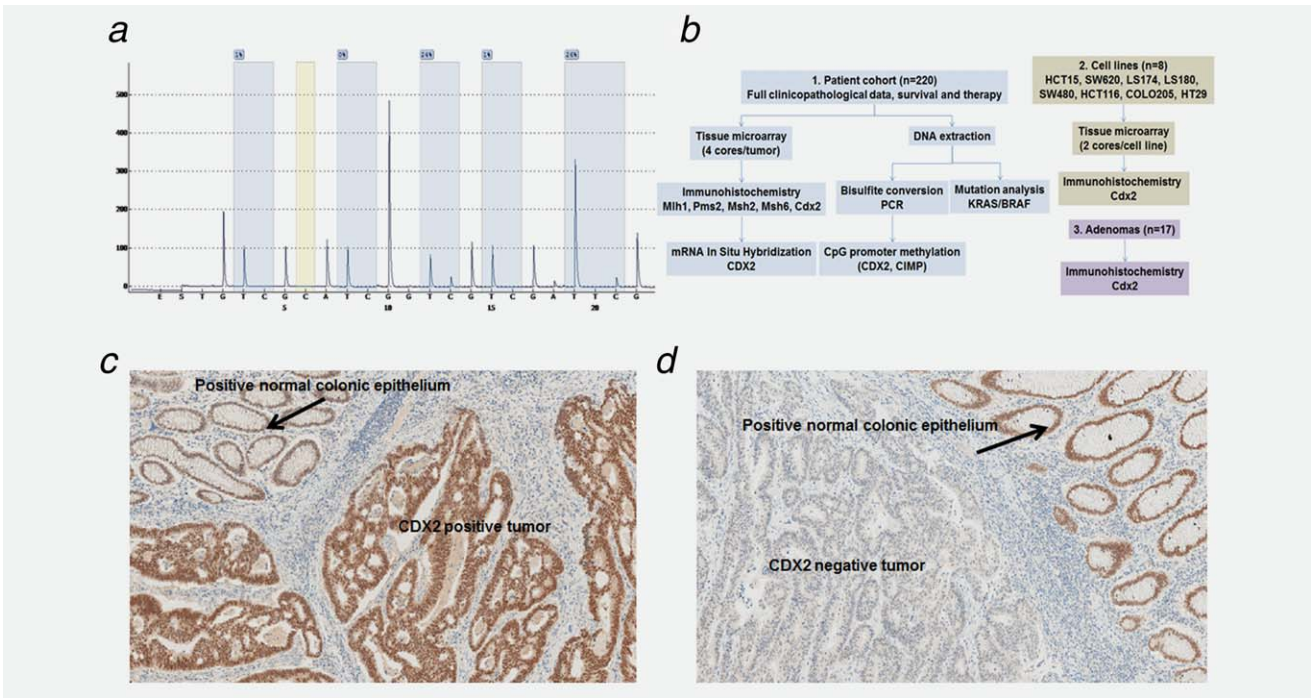

Figure 1. (*a*) Representative pyrogram of CDX2 CpG methylation assay. (*b*) Study design. (*c*) Cdx2 positive colorectal cancer with positive internal control. (*d*) Cdx2 negative cancer with positive internal control. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

carried out using the QIAxcel system (Qiagen). In preparation for pyrosequencing, immobilization of PCR products by binding of biotin to streptavidin coated Sepharose beads (GE Healthcare) was performed. A Master Mix containing a minimum of 2 µl/sample of beads, 40 µl/sample of binding buffer and 18–33 µl/sample of high-purity water was added to 5–20 µl PCR product into a PCR plate to a total volume of 80 µl. The plate was shaken for 10 minutes at room temperature. About 25 µl of 0.3 µM sequencing primer was pipetted onto a PyroMark Q24 Plate. Both PCR and Q24 plates were placed into their designated positions on the vacuum workstation. With the workstation pump on, the vacuum tool was used for 15 sec to capture beads with PCR product then lowered into 70% ethanol for 5 sec, denaturation solution for 5 sec and wash buffer for 10 sec. The vacuum tool was aligned with the Q24 plate containing the sequencing primer then switched off to allow for single stranded PCR products to make contact with sequencing primers. Annealing of sequencing primers to DNA strands was performed by heating the Q24 plate for 2 min on a heating block at 80°C. After cooling for 5 min, the plate was inserted into the PyroMark Q24 instrument for pyrosequencing. Bisulfite controls were included into the program for each assay manually to ensure complete conversion of DNA. In addition, a control oligo and water control was used in every pyrosequencing run. For each assay, the ratio of C:T indicating the percentage of methylated to nonmethylated C residues at each CpG site was noted. Then an average percentage of methylation across each case was determined. For each gene, the 75th-percentile was used as a cutoff to determine "hypermethylation" status. CIMP-high was defined by at least 3/6 hypermethylated genes, CIMP-low as at most 2/6 hypermethylated genes and CIMP-negative as 0/6 hypermethylated genes.

Study design

The study design is outlined in Figure 1*b*. The patient cohort was used to identify whether Cdx2 loss by immunohistochemistry on a multipunch tissue microarray and methylation analysis in primary colorectal cancers is related to poorer prognosis and more adverse clinicopathological features, MMR status, KRAS and $BRAF^{V600E}$ mutation as well as CIMP status. Additional mRNA ISH for CDX2 was performed. Inclusion of eight well-established colorectal cancer cell lines after paraffin embedding and TMA construction allowed the study of Cdx2 expression and correlation with molecular features. Additionally, 17 cases of adenomas were investigated for Cdx2 protein expression.

Statistics

The association between Cdx2 expression as continuous variable and categorical clinicopathological features including tumor location (left/rectum *vs.* right), histological subtype (nonmucinous *vs.* mucinous), tumor grade (G1/G2 *vs.* G3), pT (pT1-2 *vs.* pT3-4), pN (pN0 *vs.* >pN0), pM (pM0 *vs.* >pM0), venous invasion (V0 *vs.* >V0), lymphatic invasion (L0 *vs.* >L0), tumor budding (low-grade *vs.* high-grade), MMR status (proficient *vs.* deficient), KRAS and $BRAF^{V600E}$ (wild-type *vs.* mutation) and CIMP status (negative/low *vs.* high) was investigated using simple logistic regression analysis. Odds ratio (OR) and 95% confidence intervals (CI) were used to determine effect size. The Area under the ROC curve (AUC) was used to determine the discriminatory ability of Cdx2 expression for each feature, with values closer to 1.0 indicating a better discrimination. For the association with age and tumor size, linear regression analysis was used. The R-squared value was obtained. Cutoff for Cdx2 expression for analysis with $BRAF^{V600E}$ and CIMP status was determined using classification and regression tree analysis (CART) with a 10-fold cross-validation method. Chi-Square test was used for the relationship between Cdx2 low/high and $BRAF^{V600E}$ and/or CIMP status and for analysis of hypermethylation and clinicopathologcial features. p values <0.05 were considered statistically significant. All analyses were carried out using SAS V9.2 (The SAS Institute, Cary, NC).

Results

Patient characteristics

Patient characteristics are summarized in Table 1. Tissues from 220 patients were included for Cdx2 expression analysis using a multi-punch tissue microarray and for CDX2 methylation analysis, MMR status, BRAF mutation and CIMP status. Representative photomicrographs for Cdx2 expression are shown in Figures 1*c* and 1*d*. An overview of the degree of methylation for SOCS1, NEUROG1, MLH1, CDKN2A, CRABPIA and RUNX3 in addition to CDX2 is outlined in the heat map in Figure 2. For each gene included in the CIMP panel, the 75th-percentile was used as a threshold to consider the gene as hypermethylated. Then, a case was designated as CIMP-high when at least 3/6 genes were above this threshold.

Cdx2 expression and clinicopathological features

Table 2 underlines the associations between reduced Cdx2 expression within colorectal cancers and association with histomorphological, and molecular features. Reduced Cdx2 expression was significantly associated with larger tumor size ($p = 0.0154$), right-sided tumor location ($p = 0.001$), mucinous histology ($p = 0.0069$), higher tumor grade ($p < 0.0001$), more advanced pT classification ($p = 0.0234$), lymphatic invasion ($p = 0.0351$), and high-grade tumor budding ($p = 0.0131$). Moreover, MMR deficiency ($p < 0.0001$), $BRAF^{V600E}$ mutation ($p < 0.0001$) and CIMP-high status ($p = 0.0051$) were related to decreased expression of Cdx2 in tumors. There was no association with survival.

Sensitivity, specificity, positive and negative predictive values of Cdx2 for MMR-deficiency, $BRAF^{V600E}$ and CIMP-high

Using CART analysis-derived cutoff scores for Cdx2 expression, a significant loss of Cdx2 expression was considered as 90% for MMR-deficiency and ≤25% expression for $BRAF^{V600E}$ and CIMP-high. In Table 3, all 14 cases with

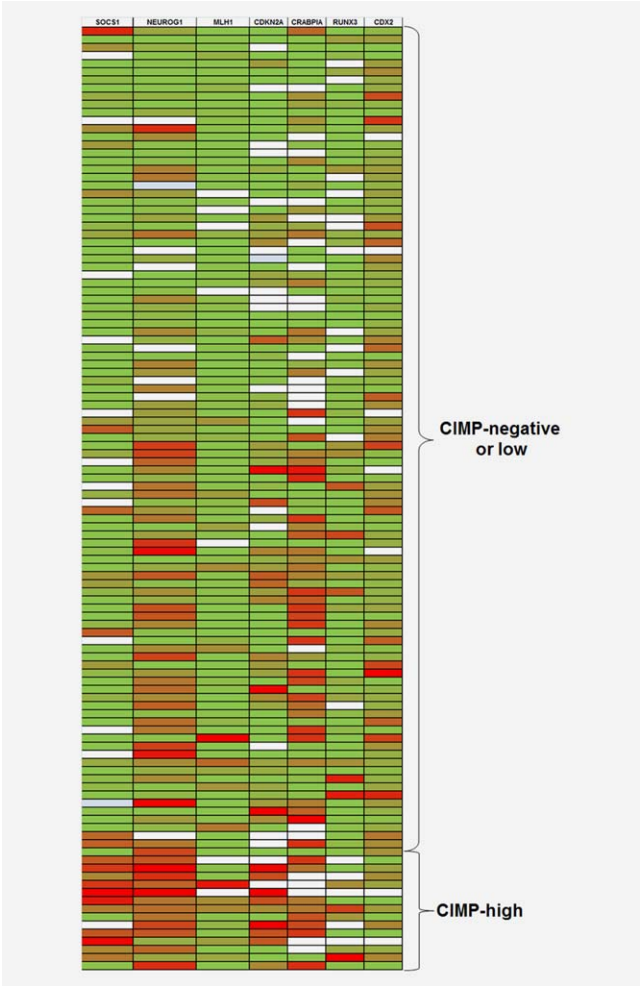

Figure 2. Heat map illustrating the degree of methylation of SOCS1, NEUROG1, MLH1, CDKN2A, CRABP1A, RUNX3, and CDX2. Red indicates higher percentages of methylation while green represents lower percentages. Empty spaces indicate non-evaluable result. For each gene in the CIMP panel to be considered hypermethylated, we used the 75th-percentile. A case was considered CIMP-H if at least 3/6 genes were hypermethylated. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

MMR-deficiency were Cdx2 low while and 100% of all Cdx2 high cases were MMR-proficient. Low and high Cdx2 was correlated with $BRAF^{V600E}$ status, CIMP status and combined BRAF/CIMP status. Of the 13 cases with low Cdx2 expression, 5 were mutated. Although the sensitivity and PPV values were 55.6 and 38.5%, the specificity and NPV for $BRAF^{V600E}$ mutation were 92.2 and 95.9%. Similar results were obtained for CIMP-high status. For patients with $BRAF^{V600E}$ mutated and simultaneous CIMP-high tumors, ($n = 5$), four could be predicted by low Cdx2 expression. Sensitivity was 80% with specificity, PPV and NPV of 91.5, 30.8, and 99%, respectively.

Cdx2 staining on a colorectal cancer cell line TMA

Immunohistochemistry for Cdx2 was additionally performed on eight colorectal cancer cell lines after embedding in paraffin and construction of a TMA. Cdx2 expression was correlated with BRAF mutation, CIMP status and MSI status. Representative photomicrographs are shown in Figure 3. CDX2 expression was completely negative in LS174, COLO205, HT29, and HCT116 but positive in HCT15, LS180, SW480, and SW680. Cdx2 negativity predicted BRAF mutation status correctly in 6/8 cell lines, CIMP-high status in 6/8 cell lines and MSI status in 4/8 cases. In a hierarchy composed of BRAF mutation, followed by CIMP-H and MSI-H, Cdx2 protein expression was predictive in all 8/8 cell lines.

Table 1. Patient characteristics ($n = 220$)

		Frequency *N* (%)
Gender	Male	102 (48.6)
	Female	108 (51.4)
Age (years)	Median (range)	71 (35–93)
Tumor size (cm)	Median (range)	4.5 (1–12)
Tumor location	Left	127 (60.5)
	Right	28 (13.3)
	Rectum	55 (26.2)
Histological subtype	Non-mucinous	185 (88.1)
	Mucinous	25 (11.9)
Tumor Grade	G1-2	132 (62.9)
	G3	78 (37.1)
pT	pT1-2	53 (25.2)
	pT3-4	157 (74.8)
pN	pN0	105 (50.0)
	pN1-2	105 (50.0)
Metastasis at diagnosis	pM0	188 (89.5)
	pM1	22 (10.5)
Venous invasion	Presence	40 (18.7)
	Absence	174 (81.3)
Lymphatic invasion	Presence	86 (40.8)
	Absence	125 (59.2)
Tumor budding	Low-grade	114 (53.8)
	High-grade	98 (46.2)
Adjuvant therapy	None	76 (35.5)
	Treated	138 (64.5)
MMR status	Proficient	196 (91.6)
	Deficient	18 (8.4)
KRAS	Wild-type	142 (67.0)
	Mutation	70 (33.0)
BRAF	Wild-type	186 (90.3)
	V600E mutation	20 (9.7)
CIMP	Negative/low	102 (87.9)
	High	14 (12.1)
Survival time (months)	Median (95%CI)	60 (48–65)

Table 2. Association of Cdx2 expression in tumor and clinicopathological features

	OR (95%CI)	*p* value	AUC	Comment
Sex	0.993 (0.981–1.006)	0.2747	0.534	
Age[1]	0.034 ± 0.036	0.3514	0.0043[2]	
Tumor size[1]	−0.015 ± 0.006	0.0154	0.03[2]	Loss of Cdx2 correlated with larger tumor diameters
Tumor location	0.978 (0.965–0.992)	0.0014	0.652	Loss of Cdx2 in right-sided tumors
Histological subtype	0.977 (0.961–0.994)	0.0069	0.668	Loss of Cdx2 in tumors with mucinous histology
Tumor grade	0.969 (0.955–0.983)	<0.0001	0.664	Loss of Cdx2 in G3 *vs*. G1-2 tumors
pT classification	0.98 (0.964–0.997)	0.0234	0.605	Loss of Cdx2 in pT3-4 *vs*. pT1-2 tumors
pN classification	0.99 (0.977–1.003)	0.1156	0.557	
pM classification	1.006 (0.985–1.029)	0.5622	0.554	
Venous invasion	0.994 (0.978–1.011)	0.4953	0.555	
Lymphatic invasion	0.987 (0.974–0.999)	0.0351	0.568	Loss of Cdx2 in L+ *vs*. L- tumors
Tumor budding	0.984 (0.972–0.997)	0.0131	0.582	Loss of Cdx2 in high-grade tumor budding cases
MMR status	0.961 (0.942–0.98)	<0.0001	0.766	Loss of Cdx2 in MMR-deficient cancers
KRAS status	0.996 (0.983–1.009)	0.5517	0.569	
BRAF status	0.961 (0.943–0.979)	<0.0001	0.734	Loss of Cdx2 in BRAF mutated tumors
CIMP status	0.967 (0.944–0.991)	0.0069	0.718	Loss of Cdx2 in CIMP-high tumors

[1]Linear regression analysis was performed. Parameter estimates with SE.
[2]*R*-squared value is shown.

Table 3. Sensitivity, specificity, positive and negative predictive values (PPV, NPV) of CDX2 for BRAF mutation, CIMP-high, BRAF mutation/CIMP-high status

		CDX2						
		Low	High	*p* value	Sensitivity	Specificity	PPV	NPV
MMR status	Proficient	30 (68.2)	48 (100.0)	<0.0001	100%	61.5%	31.8	100%
	Deficient	14 (31.8)	0 (0.0)					
BRAF	Wild-type	8 (61.5)	94 (95.9)	<0.0001	55.6%	92.2%	38.5%	95.9%
	V600E mutation	5 (38.5)	4 (4.1)					
CIMP	Negative/low	8 (61.5)	90 (91.8)	0.0076	38.5%	91.8%	38.5%	91.8%
	High	5 (38.5)	8 (8.2)					
BRAF/CIMP	WT or N/L	9 (69.2)	97 (99.0)	<0.0001	80%	91.5%	30.8%	99%
	V600E mutation and high	4 (30.8)	1 (1.0)					

CDX2 hypermethylation and clinicopathological features

Methylation analysis was successfully performed in 181 cases (Table 4). Hypermethylation was observed in 15/181 (8.3%) tumors. Although not associated with any clinicopathological feature, methylation was significantly more frequent in $BRAF^{V600E}$ mutated cancers. In addition, hypermethylation of CDX2 was strongly related to low Cdx2 protein expression and found in 28.6% of cases in comparison to only 6.1% of negative/low methylated tumors ($p = 0.0028$).

CDX2 protein expression, RNA expression and DNA methylation

The percentage of Cdx2 protein expression and CDX2 hypermethylation is shown in Supporting Information Figure 1*a* for each case. The graph outlines an inverse relationship between the two variables. Particularly, tumors with <20% Cdx2 protein expression seem to discriminate more from less heavily methylated cases. The difference in the average methylation in cases with <20% or ≥20% protein expression was significant ($p = 0.0073$; average methylation 18% *vs*. 6.3%, respectively). The ROC curve underlining these findings is shown in Supporting Information Figure 1*b* and suggests that a possible threshold of 20% for methylation may best retain its specificity for protein expression loss at <20% as well.

To resolve any discrepancy between DNA methylation and protein expression, additional RNA ISH for CDX2 was performed on the patient tissue microarray. Four hundred and eighty five tumor spots could be evaluated for RNA and

	BRAF	KRAS	CIMP	MSI	Cdx2	Correct prediction of either BRAF mutation, CIMP-high or MSI-high using Cdx2?
HCT15	wt	mut	high	high	Positive	Yes (BRAF wt)
LS174	wt	mut	negative	high	Negative	Yes (BRAF wt but MSI-high)
COLO205	mut	wt	high	stable	Negative	Yes (BRAF mut)
HT29	mut	wt	high	stable	Negative	Yes (BRAF mut)
LS180	wt	mut	negative	high	Positive	Yes (BRAF wt)
SW480	wt	mut	negative	stable	Positive	Yes (BRAF wt)
SW620	wt	mut	negative	stable	Positive	Yes (BRAF wt)
HCT116	wt	mut	high	high	Negative	Yes (BRAF wt but CIMP-high)

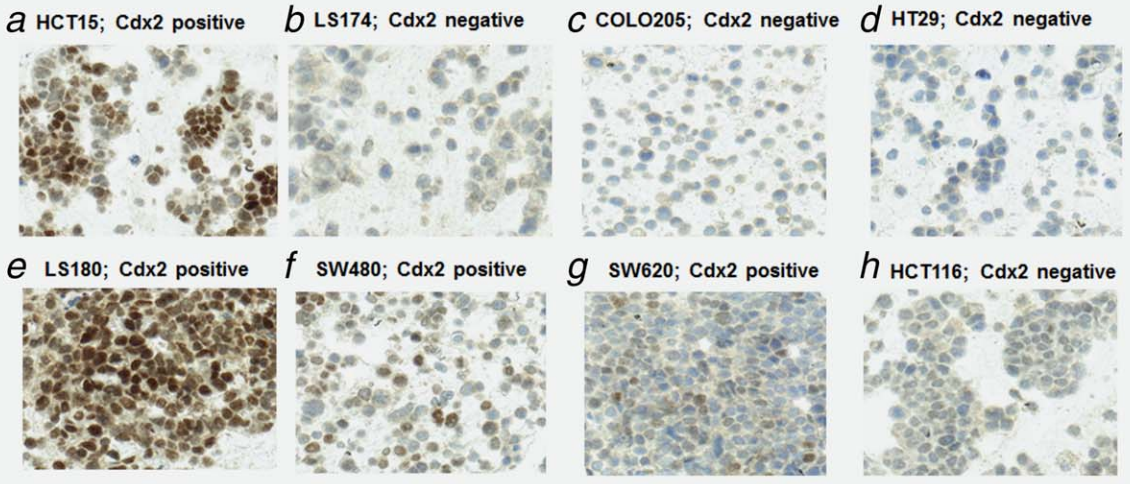

Figure 3. Cdx2 expression in a tissue microarray composed of eight colorectal cancer cell lines. The table outlines the different cell lines with molecular features and Cdx2 expression. Panels *a–h* include images of each cell line and Cdx2 expression, by immunohistochemistry (×200). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

protein (Supporting Information Fig. 2). The correlation coefficient was $r = 0.41$ ($p < 0.0001$) indicating a relatively strong positive relationship between protein and gene expression in tumor samples. Of the 80 tissue spots with <20% Cdx2 protein expression, 38 (47.5%) had an RNA ISH score = 0, 28 (35%), had a score of 1–2 and only 14 (17.5%) had a score of at least 3 ($p < 0.0001$). Of the 23 spots with 0% Cdx2 protein expression 20 had a simultaneous RNA ISH score = 0.

Comparing RNA ISH scores and DNA methylation (n = 139 evaluable samples), an inverse, albeit weak correlation was observed ($r = -0.11$). Using 20% methylation as a cut-off value, the correlation with RNA ISH was made. Again, an inverse and significant association between methylation and RNA expression was noted with a higher RNA score more frequently seen in cases with CDX2 methylation (p = 0.0477).

Cdx2 expression in SSA and TA

To obtain better insight into the timing of Cdx2 protein expression loss, 17 adenoma cases were retrieved including included 7 SSAs, 9 TAs with low-grade dysplasia, and 1 mixed case of TA with a small focus of high-grade dysplasia (5%)/SSA. Representative images are found in Supporting Information Figure 3. All cases showed diffuse staining for Cdx2 (>85% immunoreactive nuclei). However, the intensity of Cdx2 staining in some SSAs was weaker in comparison to TAs. This is especially highlighted by the case of mixed TA/SSA showing a marked loss of Cdx2 intensity in the adjacent focus of SSA.

Discussion

The novel findings of this study suggest that loss of Cdx2 expression may be involved in the serrated pathway to colorectal cancer, as demonstrated by the high specificity for $BRAF^{V600E}$, CIMP-high and MMR-deficiency.

In a first step, we investigated the loss of Cdx2 and clinicopathological features with the aim of validating previous findings. Cdx2 loss was associated with more aggressive tumor features including higher tumor grade, more advanced pT classification, lymphatic invasion, and tumor budding. Several studies underline similar correlations. In particular, Ogino *et al.* using a tissue microarray of 621 patients show a strong association of Cdx2 loss with female gender, right-sided tumor location, advanced TNM stage, high tumor grade, and mucinous or signet ring cell component.[31] A previous study from our group on more than 1,000 patients underlines these findings further with a loss of Cdx2

Table 4. Association of Cdx2 methylation and clinicopathological features

		Methylation		
		<20%	≥20%	*p* value
Gender	Male	80 (49.7)	6 (40.0)	0.5923
	Female	81 (50.3)	9 (60.0)	
Age (years)	Mean ± SD	67.6 ± 11.1	69.1 ± 13.0	0.3081
Size (cm)	Mean ± SD	4.6 ± 1.9	4.7 ± 2.6	0.9028
Tumor location	Left	92 (57.1)	11 (73.3)	
	Right	21 (13.0)	2 (13.3)	0.3815
	Rectum	48 (29.8)	2 (13.3)	
Histological subtype	Non-mucinous	140 (86.4)	15 (100.0)	0.2217
	Mucinous	22 (13.6)	0 (0.0)	
Tumor grade	G1-2	103 (63.6)	10 (66.7)	1.0
	G3	59 (36.4)	5 (33.3)	
pT	pT1-2	42 (25.9)	4 (26.7)	1.0
	pT3-4	120 (74.1)	11 (73.3)	
pN	pN0	80 (49.4)	6 (40.0)	0.4867
	pN1-2	82 (50.6)	9 (60.0)	
pM	pM0	143 (88.8)	12 (80.0)	0.3946
	pM1	18 (11.2)	3 (20.0)	
Venous invasion	Presence	30 (18.2)	4 (26.7)	0.4887
	Absence	135 (81.8)	11 (73.3)	
Lymphatic invasion	Presence	65 (40.1)	8 (53.3)	0.4127
	Absence	97 (59.9)	7 (46.7)	
Tumor budding	Low-grade	92 (56.4)	10 (66.7)	0.4436
	High-grade	71 (43.6)	5 (33.3)	
MMR status	Proficient	152 (92.1)	12 (80.0)	0.1349
	Deficient	13 (7.9)	3 (20.0)	
KRAS	Wild-type	109 (66.5)	11 (73.3)	0.7762
	Mutation	55 (33.5)	4 (26.7)	
BRAF	Wild-type	150 (92.0)	11 (73.3)	0.0184
	V600E Mutation	13 (8.0)	4 (26.7)	
CIMP	Negative/low	89 (88.9)	8 (100.0)	0.5942
	High	12 (11.2)	0 (0.0)	
Cdx2 tumor	Low (<20%)	10 (6.1)	4 (28.6)	0.0028
	High (≥20%)	153 (93.9)	10 (71.4)	

expression associated with pT, pN, tumor grade, vascular invasion, and right-sided location.[30]

It has been previously demonstrated that SSAs, implicated as precursor lesions of the serrated pathway, display loss of Cdx2 expression and often present with a gastric phenotype.[35] Such gastric differentiation may be the morphological correlate of Cdx2 loss as an intestinal transcription factor. Moreover, Cdx2 has been found to be methylated in SSAs,[36] however, to our knowledge, no correlation between methylation status and protein expression was made. We performed Cdx2 immunostaining on a small set consisting of 10 tubular and/or tubulovilllous adenomas and 7 SSAs. As expected, Cdx2 was strongly expressed in all tubular adenomas. We were not able to reproduce the findings of Mochizuka *et al.* as all SSAs displayed diffuse Cdx2 staining but interestingly, we were able to observe distinctly weaker staining of some SSAs in comparison to adenomas, nicely highlighted in the case of a mixed TA/SSA. This suggests that loss of Cdx2 may occur after the development of SSA and prior to progression to carcinoma with loss of staining intensity perhaps reflecting a "transitory" state. Whether Cdx2 itself is directly and causally involved or may

act as a surrogate marker for these molecular changes requires further elucidation.

In relation to molecular features, our results highlight significant and specific associations of Cdx2 loss with MMR-deficiency, $BRAF^{V600E}$ mutation and CIMP-high. These findings agree with Baba and colleagues who demonstrate that negative Cdx2 expression was associated with BRAF mutation (64% mutated *vs*. 23% wild-type), MSI (57% MSI-high *vs*. 23 and 34% MSS and MSI-low) and CIMP-high (67% CIMP-high *vs*. 16 and 28% CIMP-negative and low).[31] Walsh *et al*. recently showed that reduced Cdx2 was very strongly related to CIMP positivity (OR = 5.2), $BRAF^{V600E}$ mutation (OR = 5.1), MLH1 methylation (OR = 14.6) and MMR-deficiency (OR = 5.9) but not with KRAS mutation.[37] Previous work by our group on more than 300 colorectal cancers identified loss of Cdx2 expression in 23/24 $BRAF^{V600E}$ mutated cases.[32] In CIMP-high cancers, average Cdx2 expression was 35% in comparison to >80% in CIMP-negative and –low cancers. As seen by Walsh, there was no association with KRAS mutation.[37] Here, we find that loss of Cdx2 is specific although not limited to MMR-deficient cancers. Looking at recent data from the Cancer Genome Atlas Network, hypermethylation and CIMP-H phenotype includes, but is not restricted to MSI positive colorectal cancers.[38] Combined analysis of $BRAF^{V600E}$/CIMP identified Cdx2 loss as sensitive (80%) and specific (91.5%) for mutation/high. These results were again confirmed after analysis of Cdx2 expression in eight well-established cell lines. Cdx2 expression could correctly predict BRAF and CIMP status in 6/8 tumors, each. In the hierarchy of BRAF mutation, CIMP-high and MSI-H, loss of CDX2 was predictive in all eight cell lines.

This study is also in line with the recent work by Melo *et al*. who use a 146-gene classifier to identify three colorectal cancer subgroups CCS1, CCS2, and CCS3.[39] They not only find an overrepresentation of BRAF mutated, MSI/CIMP+ cases in CCS3 but show evidence from SSAs, tubular adenomas and other adenoma type suggesting that these CCS3 tumors may derive from the serrated pathway. Moreover, they illustrate complete negative immunohistochemistry staining for Cdx2 in tumors from CCS3.

We further investigated CDX2 hypermethylation as a possible cause of protein loss. Indeed, a significantly larger number of patients showed Cdx2 loss in hypermethylated cancers (29% *vs*. 6% in negative cases). Despite statistical significance however, the Cdx2 low expression in 10 out of the 14 remaining tumors are not explained by hypermethylation. Moreover, we evaluated the RNA expression of CDX2 by ISH and correlated gene expression with both methylation and protein expression. A strong positive correlation between protein and RNA expression was observed by performing a spot-by-spot comparison across 485 tumor spots ($p < 0.0001$). The expected negative correlation between methylation and RNA expression was observed, but was only weak in comparison. Taken together these results seem to underline that DNA methylation of CDX2 may be only one of several mechanisms explaining the loss of Cdx2 protein expression. Other mechanisms may exists such as loss of heterozygosity (LOH),[40] gene locus amplification[41] and MSI[42] which have all been reported to regulate expression of CDX2. Additionally, CDX2 hypermethylation was related to $BRAF^{V600E}$ but not to CIMP suggesting that methylation of CDX2 may not be related to a more global methylator phenotype. However, a more thorough investigation of CpG sites needs to be conducted in order to exclude this hypothesis.

Because the assessment of CIMP for colorectal cancers has yet to be standardized, our study may be limited by the definition of CIMP used herein. Six genes, namely SOCS1, NEUROG1, MLH1, CRABP1A, CDKN2A, and RUNX3 which are standardly included into CIMP gene-panels were used for the determination of CIMP status.[20,43] Using a strict criterion for CIMP status, 12% of patients were CIMP-high. This is on the lower end of the expect range[19,44] but consistent with other European cohorts.[7,13]

To conclude, Cdx2 may play a role in the serrated pathway to colorectal cancer as evidenced by strong relationships with $BRAF^{V600E}$, CIMP-high and MMR-deficiency. Understanding the mechanisms leading to Cdx2 loss could help to elucidate whether Cdx2 is directly and functionally involved in the progression of tumors through this pathway.

References

1. Toyota M, Ahuja N, Ohe-Toyota M, et al. CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci USA* 1999;96:8681–6.
2. Strathdee G, Appleton K, Illand M, et al. Primary ovarian carcinomas display multiple methylator phenotypes involving known tumor suppressor genes. *Am J Pathol* 2001;158:1121–7.
3. Toyota M, Ahuja N, Suzuki H, et al. Aberrant methylation in gastric cancer associated with the CpG island methylator phenotype. *Cancer Res* 1999;59:5438–42.
4. Ueki T, Toyota M, Sohn T, et al. Hypermethylation of multiple genes in pancreatic adenocarcinoma. *Cancer Res* 2000;60:1835–9.
5. Curtin K, Slattery ML, Samowitz WS. CpG island methylation in colorectal cancer: past, present and future. *Patholog Res Int* 2011;2011:902674.
6. Bae JM, Kim MJ, Kim JH, et al. Differential clinicopathological features in microsatellite instability-positive colorectal cancers depending on CIMP status. *Virchows Arch* 2011;459:55–63.
7. Dahlin AM, Palmqvist R, Henriksson ML, et al. The role of the CpG island methylator phenotype in colorectal cancer prognosis depends on microsatellite instability screening status. *Clin Cancer Res* 2010;16:1845–55.
8. Han SW, Lee HJ, Bae JM, et al. Methylation and microsatellite status and recurrence following adjuvant FOLFOX in colorectal cancer. *Int J Cancer* 2013;132:2209–16.
9. Kim JH, Shin SH, Kwon HJ, et al. Prognostic implications of CpG island hypermethylator phenotype in colorectal cancers. *Virchows Arch* 2009;455:485–94.
10. Zlobec I, Bihl MP, Foerster A, et al. Stratification and prognostic relevance of Jass's molecular classification of colorectal cancer. *Front Oncol* 2012;2:7.
11. Yamauchi M, Lochhead P, Morikawa T, et al. Colorectal cancer: a tale of two sides or a continuum? *Gut* 2012;61:794–7.
12. Ogino S, Odze RD, Kawasaki T, et al. Correlation of pathologic features with CpG island methylator phenotype (CIMP) by quantitative DNA methylation analysis in colorectal carcinoma. *Am J Surg Pathol* 2006;30:1175–83.

13. Zlobec I, Bihl M, Foerster A, et al. Comprehensive analysis of CpG island methylator phenotype (CIMP)-high, -low, and -negative colorectal cancers based on protein marker expression and molecular features. *J Pathol* 2011;225:336–43.
14. Iacopetta B, Kawakami K, Watanabe T. Predicting clinical outcome of 5-fluorouracil-based chemotherapy for colon cancer patients: is the CpG island methylator phenotype the 5-fluorouracil-responsive subgroup? *Int J Clin Oncol* 2008;13:498–503.
15. Samowitz WS, Albertsen H, Sweeney C, et al. Association of smoking, CpG island methylator phenotype, and V600E BRAF mutations in colon cancer. *J Natl Cancer Inst* 2006;98:1731–8.
16. Jover R, Nguyen TP, Perez-Carbonell L, et al. 5-Fluorouracil adjuvant chemotherapy does not increase survival in patients with CpG island methylator phenotype colorectal cancer. *Gastroenterology* 2011;140:1174–81.
17. Min BH, Bae JM, Lee EJ, et al. The CpG island methylator phenotype may confer a survival benefit in patients with stage II or III colorectal carcinomas receiving fluoropyrimidine-based adjuvant chemotherapy. *BMC Cancer* 2011;11:344.
18. Van Rijnsoever M, Elsaleh H, Joseph D, et al. CpG island methylator phenotype is an independent predictor of survival benefit from 5-fluorouracil in stage III colorectal cancer. *Clin Cancer Res* 2003;9:2898–903.
19. Ogino S, Nosho K, Kirkner GJ, et al. CpG island methylator phenotype, microsatellite instability, BRAF mutation and clinical outcome in colon cancer. *Gut* 2009;58:90–6.
20. Weisenberger DJ, Siegmund KD, Campan M, et al. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nat Genet* 2006;38:787–93.
21. Kriegl L, Neumann J, Vieth M, et al. Up and downregulation of p16(Ink4a) expression in BRAF-mutated polyps/adenomas indicates a senescence barrier in the serrated route to colon cancer. *Mod Pathol* 2011;24:1015–22.
22. Shima K, Nosho K, Baba Y, et al. Prognostic significance of CDKN2A (p16) promoter methylation and loss of expression in 902 colorectal cancers: cohort study and literature review. *Int J Cancer* 2011;128:1080–94.
23. Hawkins N, Norrie M, Cheong K, et al. CpG island methylation in sporadic colorectal cancers and its relationship to microsatellite instability. *Gastroenterology* 2002;122:1376–87.
24. Leggett B, Whitehall V. Role of the serrated pathway in colorectal cancer pathogenesis. *Gastroenterology* 2010;138:2088–100.
25. Carragher LA, Snell KR, Giblett SM, et al. V600EBraf induces gastrointestinal crypt senescence and promotes tumour progression through enhanced CpG methylation of p16INK4a. *EMBO Mol Med* 2010;2:458–71.
26. Minoo P, Moyer MP, Jass JR. Role of BRAF-V600E in the serrated pathway of colorectal tumourigenesis. *J Pathol* 2007;212:124–33.
27. Rad R, Cadinanos J, Varela LRI, et al. A genetic progression model of BrafV600E-induced intestinal tumorigenesis reveals targets for therapeutic intervention. *Cancer Cell* 2013;24:15–29.
28. Li MK, Folpe AL. CDX-2, a new marker for adenocarcinoma of gastrointestinal origin. *Adv Anat Pathol* 2004;11:101–5.
29. Werling RW, Yaziji H, Bacchi CE, et al. CDX2, a highly sensitive and specific marker of adenocarcinomas of intestinal origin: an immunohistochemical survey of 476 primary and metastatic carcinomas. *Am J Surg Pathol* 2003;27:303–10.
30. Lugli A, Tzankov A, Zlobec I, et al. Differential diagnostic and functional role of the multi-marker phenotype CDX2/CK20/CK7 in colorectal cancer stratified by mismatch repair status. *Mod Pathol* 2008;21:1403–12.
31. Baba Y, Nosho K, Shima K, et al. Relationship of CDX2 loss with molecular features and prognosis in colorectal cancer. *Clin Cancer Res* 2009;15:4665–73.
32. Zlobec I, Kovac M, Erzberger P, et al. Combined analysis of specific KRAS mutation, BRAF and microsatellite instability identifies prognostic subgroups of sporadic and hereditary colorectal cancer. *Int J Cancer* 2010;127:2569–75.
33. Karamitopoulou E, Zlobec I, Kolzer V, et al. Proposal for a 10-high-power-fields scoring method for the assessment of tumor budding in colorectal cancer. *Mod Pathol* 2013;26:295–301.
34. Karamitopoulou E, Lugli A, Panayiotides I, et al. Systematic assessment of protein phenotypes characterizing high-grade tumour budding in mismatch repair-proficient colorectal cancer. *Histopathology* 2010;57:233–43.
35. Mochizuka A, Uehara T, Nakamura T, et al. Hyperplastic polyps and sessile serrated 'adenomas' of the colon and rectum display gastric pyloric differentiation. *Histochem Cell Biol* 2007;128:445–55.
36. Dhir M, Yachida S, Van Neste L, et al. Sessile serrated adenomas and classical adenomas: an epigenetic perspective on premalignant neoplastic lesions of the gastrointestinal tract. *Int J Cancer* 2011;129:1889–98.
37. Walsh MD, Clendenning M, Williamson E, et al. Expression of MUC2, MUC5AC, MUC5B, and MUC6 mucins in colorectal cancers and their association with the CpG island methylator phenotype. *Mod Pathol* 2013, doi: 10.1038/modpathol.2013.101. [Epub ahead of print].
38. Cancer Genome Atlas Network, Comprehensive molecular characterization of human colon and rectal cancer. *Nature* 2012;487:330–7.
39. De Sousa EMF, Wang X, Jansen M, et al. Poor-prognosis colon cancer is defined by a molecularly distinct subtype and develops from serrated precursor lesions. *Nat Med* 2013;19:614–8.
40. Sivagnanasundaram S, Islam I, Talbot I, et al. The homeobox gene CDX2 in colorectal carcinoma: a genetic analysis. *Br J Cancer* 2001;84:218–25.
41. Subtil C, Guerin E, Schneider A, et al. Frequent rearrangements and amplification of the CDX2 homeobox gene in human sporadic colorectal cancers with chromosomal instability. *Cancer Lett* 2007;247:197–203.
42. Ishikawa T, Fujita T, Suzuki Y, et al. Tumor-specific immunological recognition of frameshift-mutated peptides in colon cancer with microsatellite instability. *Cancer Res* 2003;63:5564–72.
43. Ogino S, Kawasaki T, Kirkner GJ, et al. Evaluation of markers for CpG island methylator phenotype (CIMP) in colorectal cancer by a large population-based sample. *J Mol Diagn* 2007;9:305–14.
44. van Rijnsoever M, Grieu F, Elsaleh H, et al. Characterisation of colorectal cancers showing hypermethylation at multiple CpG islands. *Gut* 2002;51:797–802.